SPERM FILTRATES AND DIALYZATES.

Their Action on Ova of the Same Species.[1]

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Introduction.

The idea persists that spermatozoa bear a specific substance which is essential in fertilization. Yet all attempts to extract from spermatozoa a substance which will cause development of ova of the same species have failed or have been open to grave criticism. It is now possible to avoid certain errors which invalidated the results of these investigations. The experiments, to be reported here, were undertaken to determine whether, by processes of filtration and dialysis, solutions can be obtained from living sperm which will effect activation and development of ova of the same species. For interesting me in this problem and for invaluable aid in its solution I am indebted to Dr. Otto C. Glaser.

The experimental work was carried on during the summers of 1919–1922 at the Marine Biological Laboratory at Woods Hole, Massachusetts, and from December until June 1920–1921 at the

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Hopkins Marine Laboratory at Pacific Grove, California. I wish to express my appreciation of the hospitality extended to me at these laboratories, and my thanks to their Directors, Dr. Frank R. Lillie and Dr. Walter K. Fisher, for their aid and encouragement.

The fact that the spermatozoa, rather than the medium which carries them, are the essential agents in fertilization was established in 1824 by Prevost and Dumas; and it is now conceded that fertilization is monospermic and involves the combination of nuclear material from a single spermatozoön and a single ovum. In many species only a single spermatozoön penetrates the cytoplasm of the egg, as was shown by Hertwig (1876); but even when in normal fertilization more than one enters the egg, as in the pigeon, the pro-nucleus of only one enters into the formation of the fertilization nucleus (Harper, 1904, and Blount, 1909). As stated by Glaser (1915), "a single spermatozoön is sufficient to carry on the biparental effect."

It is well known, however, that in the process of fertilization in any species the spermatozoa far exceed the ova in number. This was first noted in frogs by Spallanzani (1785), and verified by Prevost and Dumas (1824). Under such conditions direct contact of every ovum with a single spermatozoön is possible. But mere contact does not ensure fertilization. Even in physiological solutions spermatozoa rapidly lose their fertilizing power, Vernon (1899); Gemmil (1900); Dungay (1913); Lillie (1915) and Cohn (1918). Two explanations have been offered in recent years to account for the excess of spermatozoa. Lillie (1915) implies that in an excess at least one spermatozoön *possessing sufficient fertilizing power* ("sperm receptors") will reach each ovum. On the other hand Glaser (1915) and Cohn (1918) state that the extra spermatozoa may produce changes in an ovum which facilitate the inclusion of a single spermatozoön.

The idea of a superficial effect of sperm on ova is supported by results obtained with *Nereis limbata*, Lillie (1911) and Goodrich (1920); with *Arbacia punctulata*, Lillie (1921); with *Sabellaria alveolata* and *Halosydna gelatinosa*, Labbe (1921, 1922); and with *Asterias*, Chambers (1923). There are indications that a chemical substance or substances are produced by the sperm:

Godlewski (1911); Herlant (1912); Heilbrun (1915); and Lillie (1911, 1915, 1921).

What real evidence have we to support the suggestion, recurrent in one form or another, that the sperm exude a substance which facilitates fertilization? The early attempts of Spallanzani (*loc. cit.*), with filtrates, and of Prevost and Dumas (*loc. cit.*), with filtrates and extracts of spermatozoa, to demonstrate such a substance gave purely negative results. In later work: Pierri (1899); Winkler (1900); Du Bois (1900); Gies (1901); Pizon (1905); Jacoby (1910); Morse (1912); Robertson (1912); Foa (1918)—positive results, where reported, are actually vitiated by errors of technique or interpretation, Loeb (1913, p. 104, and 1916, p. 203); and Lillie (1919, pp. 133, 134).

II. Methods.

In an investigation of this kind so much depends upon the methods employed that it seems desirable to present these in some detail.

All glassware was cleaned with potassium-bichromate-sulphuric acid mixture, subsequently removed by washing in fresh and in sea-water. All solutions were made with analyzed chemicals (Kahlbaum's, Merck's, and Baker's) with water redistilled from glass. Specific gravity was determined in early experiments with a standard hydrometer and later with a Westphal balance. A special set of indicators and standards provided and tested at intervals by Hynson, Westcott and Dunning was used for the colorimetric determination of the hydrogen-ion concentration, Clowes and Smith (1923 and 1924). Every possible precaution was taken to avoid accidental insemination and sea-water controls accompanied every experiment.

Method of Obtaining Ripe Reproductive Cells of Sea-urchins.—One method is as follows: The instruments and hands of the operator are thoroughly washed in tap water and the animals are washed in tap water followed by sea-water. They are opened by cutting through the oral disc with sterile scissors, the disc and the alimentary canal are removed with sterile forceps and the cœlom is flushed with filtered sea-water to remove the body fluids and any intestinal contents. Males and females are

placed on opposite sides of the operator. Each animal, unless shedding freely, is immediately wiped and placed on its aboral surface in a Syracuse watch glass. In this position the reproductive cells exude through the genital pores. In a second method suggested by Dr. Glaser, the spines are rubbed off before the animals are washed and dried. If the reproductive elements are ripe, shedding begins immediately and it is unnecessary to cut the "test." There is no admixture with body fluids or with sea-water and very clean dry reproductive cells are obtained. Only ripe spermatozoa are shed and these are collected in a beaker. Not all the eggs shed are ripe. For this reason it is necessary to fertilize a sample of eggs from each female. After this the "certified" eggs are placed together in a finger bowl with sufficient sea-water to keep the concentration of egg-water below a point at which it will injure the eggs.

Method of Obtaining Ripe Eggs of Nereis limbata.—The method recommended by Lillie (1911) and Just (1915*a*) was employed. Males and females as they were caught were segregated in finger bowls of sea-water and kept cool until all preparations for an experiment were complete. The most satisfactory results are usually obtained with reproductive cells taken from such animals within two hours after collection; although, if necessary, shedding can be prevented for twelve hours or more if the dishes containing the animals are kept on ice. Since either drying the animals on filter paper or washing them in distilled water leads to shedding, each animal was "sterilized" by first transferring it to a finger bowl containing two hundred and fifty milliliters of sea-water. (In this volume of sea-water any sperm adhering to the body of the female will lose their fertilizing power.) Subsequently each animal was placed in a dry Syracuse watch glass in which any excess of moisture was absorbed with strips of filter paper, and there cut transversely with sterilized scissors. The eggs or sperm were forced out by the spasmodic contractions of the body muscles. To avoid accidental insemination the males were opened with a second set of instruments; and all inseminated controls were kept separate from the experiments in which special solutions were being tested. Eggs and sperm were kept covered and cool until used.

Preparation of Filters.—Berkefeld and Mandler diatomaceous filters were used: in preliminary experiments the coarse and medium grade of Berkefeld filters; and in all other work Mandler filters, tested to six to twelve pounds air pressure in the size two and one half by five eighths inches. The latter are used generally in bacteriological work. These were boiled in five per cent. aqueous solutions of sodium bicarbonate, washed and boiled repeatedly in redistilled water until the wash water was neutral in reaction. Finally they were thoroughly washed in filtered sea-water. After this a stream of sea-water was passed through the filters until samples of such water produced no injurious effect on unfertilized ova and no disturbance of fertilization or of development. Such filters were considered "clean." Every filter was subjected to this treatment each time that it was used. The necessity for these precautions was indicated by a variety of experiences. The first sea-water passed through a boiled and washed filter may be sufficiently hypotonic to cause cytolysis of ova within twenty-four hours. Unless all alkali is removed the filtered sea-water may produce activation of ova. The use of acid in the cleaning of filters is prohibitive because the filtrates would then contain traces of heavy metal dissolved from the filter bands. The impurities in question are often too slight for detection by chemical tests, but are only too clearly revealed by their effects upon unfertilized or fertilized ova.

Preparation of Sperm Filtrates.—Five and ten per cent. suspensions of spermatozoa were prepared by adding to a definite volume of dry sperm a measured quantity of sea-water. These were allowed to stand at room temperature (fifteen degrees centigrade at Pacific Grove and twenty-two degrees centigrade at Woods Hole) for from five minutes to four hours. To ensure the greatest activity of the spermatozoa the carbon dioxide generated by them was prevented from accumulating by a thorough aëration of the suspensions. The latter were then either clarified first by centrifuging or by filtering through filter paper, or were transferred directly to a diatomaceous filter. Contamination of the filtrates by back suction was prevented by collecting the filtrates into Pyrex test tubes set in the suction flasks.

During the passage of the filtrate it is necessary to prevent or at least to minimize the destruction of spermatozoa by dehydration or compression. This was attempted by not allowing the surface of the mantle to become exposed to air and by frequent and cautious stirring of the suspension. Some of the spermatozoa are not seriously injured in the process of filtration as indicated by the isoagglutinable and fertilizing power of the sperm remaining on the surface of the mantle at the end of the process.

The filtrates, which are automatically freed from any excess of carbon dioxide in passing through the filter mantle, are then transferred to flasks which are kept tightly plugged and placed in a refrigerator. These solutions remain free from bacteria and retain their peculiar physiological properties for at least a month.

Preparation of Sperm Dialyzates.—There is one objection to the use of sperm filtrates. Some spermatozoa may undergo destruction on the surface of the mantle. To meet this objection I prepared dialyzates of sperm in order to compare their action on ova with that of filtrates.

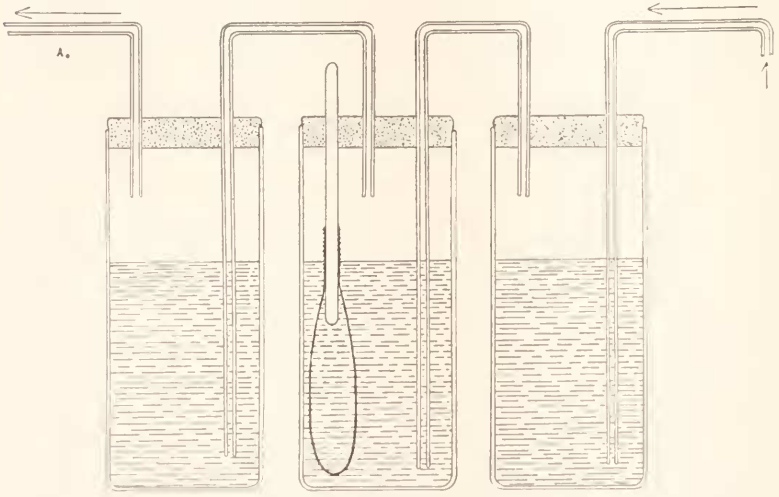


FIG. 1. Dialyzing apparatus. Air is drawn through the sea-water in the three bottles by suction exerted at *A*. The collodion sac is suspended in the sea-water in the central bottle and this sea-water dialyzate used in experiments later. The outer two bottles serve as safety flasks.

Collodion sacs used as dialyzers were made in fifty milliliter centrifuge tubes from a twelve per cent. collodion prepared

according to the method of Gates (1921). The sacs were washed in redistilled water and in sea-water. Samples of sea-water, allowed to stand in the washed sacs for at least twelve hours, were tested in the same manner as that passed through the diatomaceous filters. Tests for leakage were made and any imperfect sacs were discarded. Ten milliliters of twenty-five or of fifty per cent. suspensions of sperm were placed in a tested collodion sac and dialyzed against forty milliliters of sea-water. The resultant dialyzates may be considered comparable to filtrates prepared from five and ten per cent. suspensions of sperm. The sea-water dialyzates must be aërated constantly to provide the most favorable conditions for the sperm during the process. This involves two dangers: evaporation and contamination of the dialyzates. In order to eliminate these sources of error the method illustrated in Fig. 1 was employed.

In these experiments dialysis was continued for five to twenty-four hours. With one exception the sperm in the collodion sacs retained their fertilizing power and their capacity for iso-agglutination at the end of the process.

III. Properties of Preparations.

a. Physical and Chemical Properties.—The osmotic pressure of filtrates and dialyzates, as indicated by the specific gravity tests, is the same as that of sea-water. There is a slight variation in some preparations and in sea-water but it is not sufficient in itself, as determined by experimentation, to produce activation. The hydrogen-ion concentration of filtrates and dialyzates was also equal to that of sea-water (p_H 7.9–8.1 at Woods Hole; 7.6–7.8 at Pacific Grove), indicating that the carbon dioxide formed by active sperm was completely removed by aëration and that the sperm added no other free hydrogen-ions. There is a possibility that small amounts of acid may have combined with buffers in sea-water. The question now arises whether these preparations contain any active physiological principle. No living spermatozoa or fragments were ever observed, nor was there ever a case of normal fertilization in any of the preparations. It is also certain that they contain no chemical substance whose concentration falls within the range of sensitivity of the usual chemical methods.

Experiments in which blood plasma was passed through a Berkefeld filter, Cramer and Pringle (1913), and Goddard (1914), demonstrated that the filter held back for a time, not only suspended elements, but the various proteins in colloidal solution, but the third portion passed through the filter contained some fibrinogen. Mudd (1922) suggested that in alkaline solution protein may be carried through in small amounts. The filter itself is negatively charged and because of the amphoteric properties of protein the latter dissociates as an anion in alkaline solutions and so would tend to be carried through the filter.

It is important therefore to determine if possible whether nitrogen compounds are present since Loeb (1914) states that protamine will induce the first cell divisions in eggs of *Arbacia;* and Labbe (1923) using a one to forty thousand solution of sodium nucleinate in sea-water on unfertilized eggs of *Arbacia* obtained some swimming larvæ. Because of buffer action these solutions had the same hydrogen-ion concentration as sea-water, and Labbe concluded that the sodium nucleinate exerted a specific action not due to its hydrogen-ion concentration.

All the usual tests for protein or protein split products such as guanine, protamine, and nucleic acid were negative. A micro-kjehldahl test, performed for me by Dr. W. Dennis, was also negative, indicating that nitrogen is not present in sufficient amounts to be detected by this method. Incineration tests gave evidence of the presence of carbon in larger amounts in both filtrates and dialyzates than in sea-water.

b. Physiological Properties.—From the presence of carbon in these preparations it is certain that they contain something of organic origin. It seemed possible that they might produce changes in the ova of foreign species, since sperm extracts obtained by other investigators have produced marked effects on ova of foreign species even when they had no influence on ova of the same species, Loeb (1916, p. 102). Consequently tests were made to determine whether *Arbacia* sperm filtrates would affect the ova of *Nereis limbata*, a form in which maturation follows insemination. Seventeen experiments were performed in which ten preparations were used on *Nereis* eggs. The results obtained resemble in many respects those produced by in-

semination with *Nereis* sperm. The foreign filtrate causes production of jelly, the formation of a "fertilization membrane," complete maturation, and segmentation leading to the development of larvæ.[2] The latter however are abnormal in shape and in the distribution of cilia. In no case did these changes occur in sea-water controls. A protocol of one experiment is given below.

TABLE I.

CHANGES IN THE OVA OF *Nereis* EXPOSED FOR TWENTY MINUTES TO A FILTRATE PREPARED FROM A TWO PER CENT. SPERM SUSPENSION OF *Arbacia*.

Date.	Exper.	Per Cent. of Eggs Forming Jelly and Membranes.	Per Cent. of Dividing Eggs.	Per Cent. Swimming.
8/ 4/20	31	100	10	0.5
8/ 5/20	33	99	90	0
8/ 7/20	35	81	75	5
8/ 8/20	37	85	85	9
8/ 8/20	38	100	14.5	2
8/13/20	40	100	1	0
8/14/20	43	100	5	2
8/14/20	44	100	13.5	1
8/14/20	45	100	12	0.5
7/27/21	225	33	31.5	1
7/27/21	225	30	28.5	1

In experiments 31, 40, and 43 the ova were stale. In all cases membrane formation and maturation occurred as rapidly in ova in sperm filtrates as in inseminated ova. Subsequent development in ova treated with filtrate was delayed.

The *Arbacia* sperm filtrate acted like a parthenogenetic agent, yielding results similar to those obtained in *Nereis* with other methods (Lillie, 1911, and Just, 1915).[2] I obtained similar results with filtered egg-water of *Arbacia*. The latter observations, quoted by Dr. Alvalyn Woodward (1921), were verified frequently in subsequent experiments. Further experimentation demonstrated that the effect produced by sperm filtrates on *Nereis* eggs bears a definite relation to the strength of the preparations and to the duration of exposure of ova to them. The main question however is what effect have such filtrates and dialyzates on ova of the same species.

[2] The developing ova divided into two, four and eight cells, and subsequently some of them developed into abnormal ciliated trochophores. The rest cytolyzed.

IV. The Potency of Sperm Filtrates and Dialyzates on Ova of the Same Species.

a. Arbacia punctulata.—The procedure employed to determine the effect of these preparations on ova of the same species and the results obtained are described first for *Arbacia*. The ova were exposed to the action of sperm filtrates and dialyzates in the proportion of two milliliters of fresh washed eggs to twenty-five milliliters of the test solution. Sea-water controls accompanied every experiment and samples of eggs from test solution and from sea-water were examined at intervals.

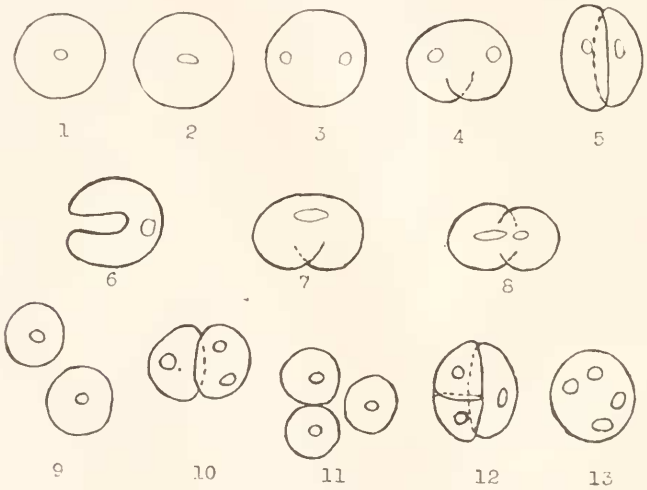
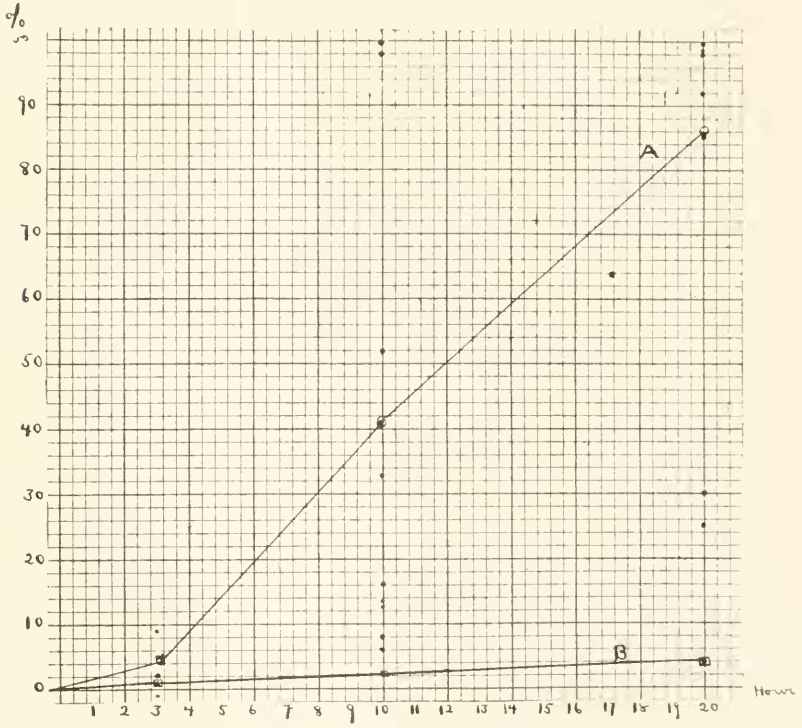


Fig. 2. Changes in shape and nuclear changes in ova exposed to sperm filtrates. 1–5 = changes in a single ovum within forty-five minutes; 6–13 = ova representing various changes produced by filtrates. Magnification × 350.

The first evidence of the influence of the filtrate or of the dialyzate on the ova is a change in the density of the cytoplasm at or near the center of the ovum, similar to that which precedes nuclear division in inseminated eggs. Indeed nuclear changes and nuclear division follow. Simultaneously a distortion of the egg occurs followed by its partial or complete cleavage into two or more parts. The cleavages are at times perfectly regular, at times unequal. Even the smallest cells are usually nucleated. Segmentation sometimes proceeds to the eight-celled stage, but in any case is followed, if the eggs remain in the filtrate, by a

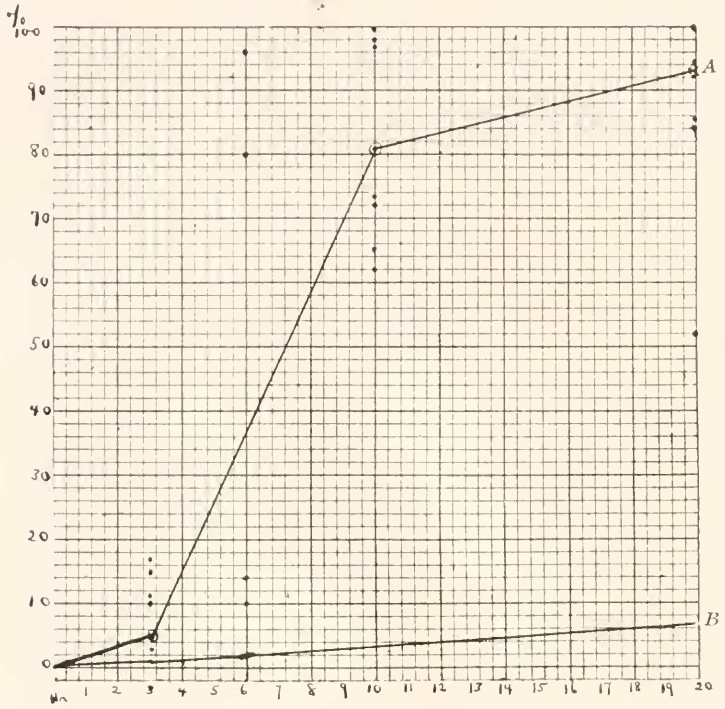
separation of the parts and their ultimate cytolysis. In a very few cases a slightly elevated membrane is visible. These results were reproduced with different lots of eggs and with different filtrates and dialyzates. In no case did these changes occur in sea-water controls or in sea-water filtered or dialyzed. The latter results indicate that no substance is given off by the filters or collodion sacs that can account for the effects of sperm filtrates and dialyzates.



GRAPH I. The percentage of ova divided and cytolyzed by sperm filtrates of the same species—*Arbacia punctulata*. *A* = the average of ten experiments with filtrates made from 2 per cent. suspensions. *B* = the average of ten sea-water controls. ● = percentages in individual experiments with sperm filtrates.

Confirmation of these results was obtained in a series of forty-two experiments with filtrates and twenty-four experiments with dialyzates. Within twenty-four hours the majority of ova in the test solutions had undergone decided changes, while those in

controls remained normal in appearance. This is well illustrated in the percentages indicated in graphs I., II., and III.[3]



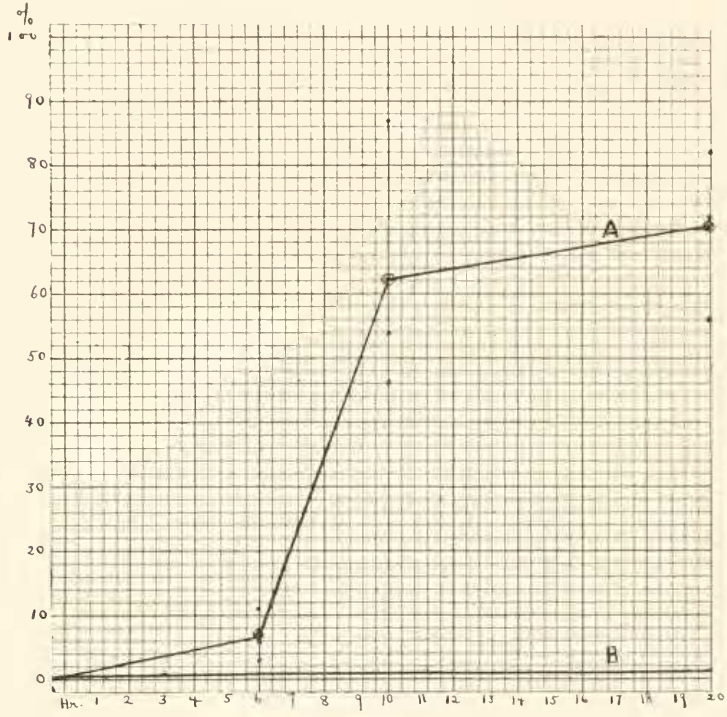
GRAPH II. The percentage of ova divided and cytolyzed by sperm filtrates of the same species—*Arbacia*. *A* = the average of ten experiments with filtrates made from 5 per cent. suspensions. *B* = the average of ten sea-water controls. ● = percentages in filtrates in individual experiments.

It is evident that some ova, probably because of a slight difference in physiological condition, are more rapidly affected by the filtrates and dialyzates than others, thus indicating a varying degree of susceptibility of the eggs to the action of the preparations.

In view of the failure of other investigators of this problem it is important to emphasize the fact that I obtained consistent results. At the same season of the year the eggs of different

[3] The graphs indicate the number divided and cytolyzed rather than simply the ones in a state of division. Since the ova do not divide simultaneously, some have divided and cytolyzed at a time when others are undergoing nuclear changes or are dividing.

females are about equally susceptible, and filtrates or dialyzates prepared in the same manner are equally effective on ova of the same individual, as indicated in graphs IV. and V.



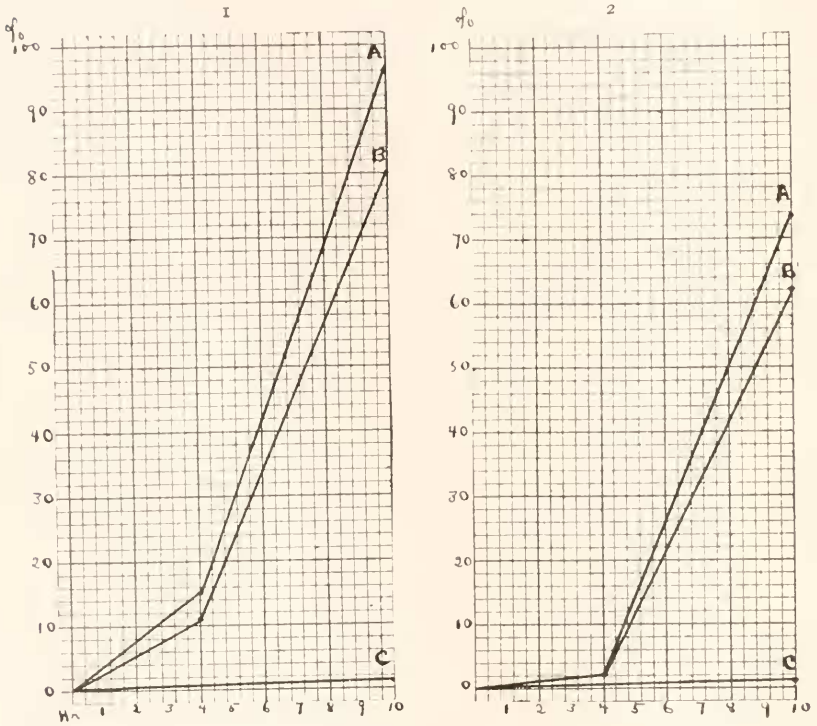
GRAPH III. The percentage of ova divided and cytolyzed in sperm dialyzates of the same species—*Arbacia punctulata*. *A* = the average of three experiments with dialyzates from 50 per cent. suspensions. Duration of dialysis = ten hours. *B* = the average of three experiments with sea-water dialyzed for ten hours against sea-water. ● = percentage in dialyzates in individual experiments.

It is apparent that, if the eggs are in the same physiological condition and are exposed at the same temperature to filtrates of equal strength, one may expect equal amounts of segmentation and cytolysis in approximately equal lengths of time.

As might be expected a definite correlation exists between the strength of the test solutions and the percentage of eggs affected in a given time. This is well illustrated in graphs VI., VII., VIII., and IX.

The definite correlation which exists between the duration of

exposure and the percentage of eggs affected suggested experiments in which the eggs are removed from the filtrate before the latter have had opportunity to produce any visible changes in the eggs. If now there are invisible effects and if these are orderly and significant, they should become noticeable after the

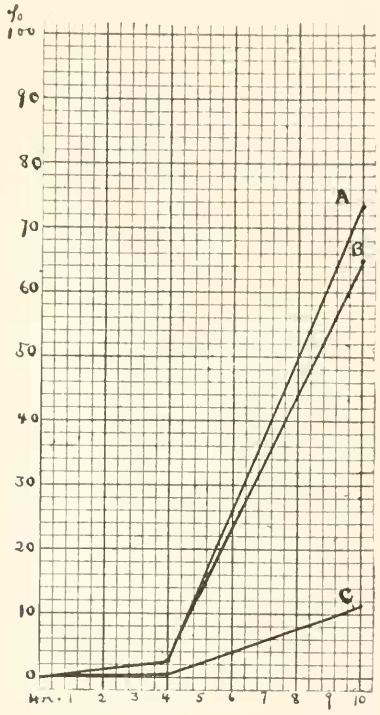


GRAPH IV. Percentage of division and cytolysis of ova produced by sperm filtrates on two sets of eggs—*Arbacia punctulata*. 1 = Filtrate 6/30/21 from a 5 per cent. suspension. 2 = Filtrate 7/2/21 from a 5 per cent. suspension. *A* = one set of eggs. *B* = second set of eggs. *C* = control eggs in S.W.

eggs are transferred to sea-water. Cases in point are illustrated in graphs X., XI., and XII., and indicate clearly that the gross visible effects, distinct only after several hours, are preceded by important changes which may develop in some of the ova within a very few minutes. These changes may, after transfer of the ova to sea-water, lead to progressive changes in the ova.

The Effect of Filtrates Prepared from Heated Sperm.—The statement was made by Winkler (1900) and by Morse (1912)

that when sperm suspensions were heated to 50°–60° centigrade the solutions failed to affect eggs. I therefore made filtrates from suspensions of *Arbacia* sperm heated to 42°–50° centigrade to compare their action with those of filtrates from unheated



GRAPH V. Percentage of ova divided and cytolyzed by two similar filtrates of sperm of the same species—*Arbacia*. *A* = Sperm filtrate 6/30/21 from a 5 per cent. suspension. *B* = Sperm filtrate 7/2/21 from a 5 per cent. suspension. *C* = Sea-water control.

sperm suspensions. The spermatozoa lose their iso-agglutinable and their fertilizing properties at a temperature of 38°–40° centigrade and tend to adhere to one another. One heated suspension was first passed through filter paper and then through a Mandler filter. As a control a part of the same suspension, unheated, was similarly treated. A comparison of the effect of the two filtrates is given in the following table.

TABLE II.

A COMPARISON OF THE ACTION OF FILTRATES PREPARED FROM HEATED AND FROM UNHEATED SPERM SUSPENSIONS OF *Arbacia* FIRST PASSED THROUGH WHATMAN FILTER PAPER.

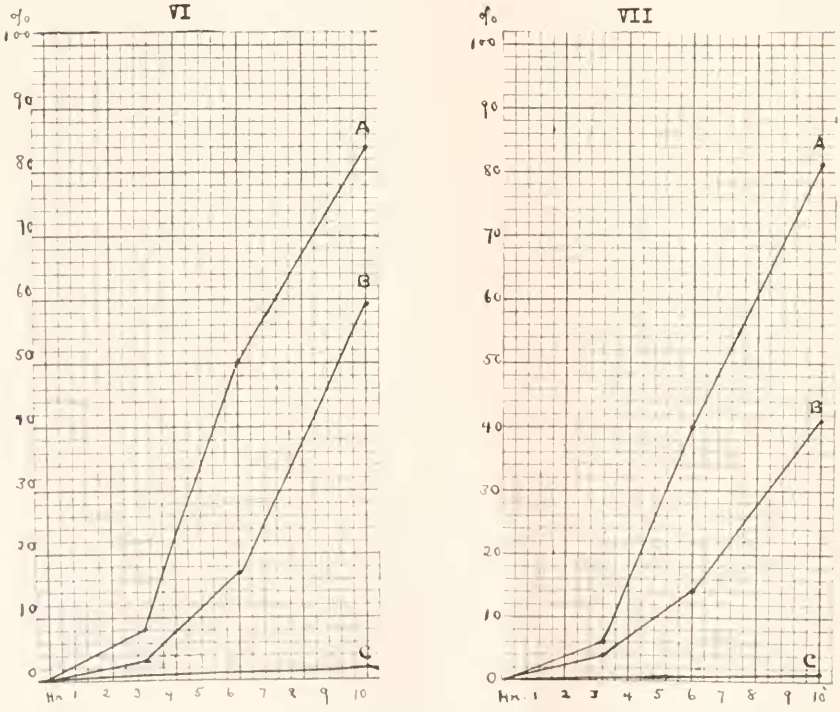
Filtrate from	Per Cent. of Ova Affected.				Per Cent. of Ova Affected.			
	4.5 Hrs.				7.5 Hrs.			
	Nor.	Abn.	Div.	Cyt.	Nor.	Abn.	Div.	Cyt.
I. Fresh Sperm	74	24.5	0.5	1	1	0	0	99
II. Heated Sperm	99	0	0	1	99	0	0	1

Nor.—normal undivided ova.

Abn.—undivided ova, abnormal in shape and in the appearance of the cytoplasm and nucleus.

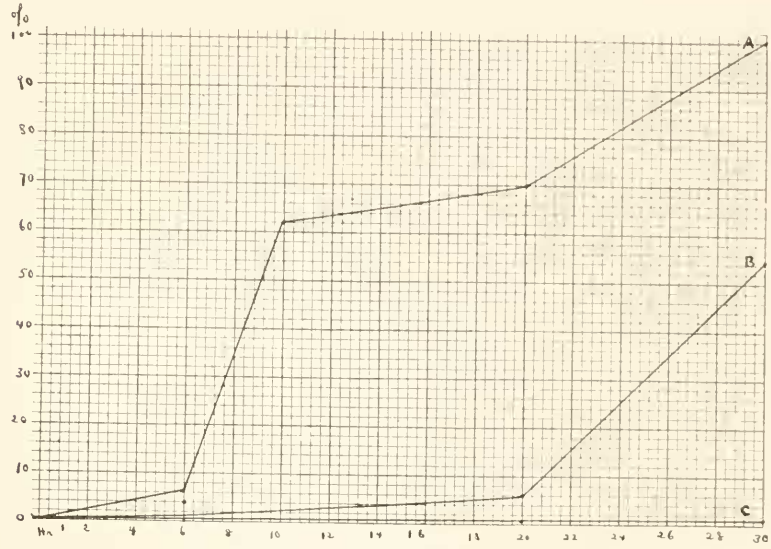
Div.—divided.

Cyt.—cytolyzed.



GRAPHS VI. AND VII. The relation of the strength of the filtrate to the percentage of ova divided and cytolyzed—*Arbacia*. VI. Average of four experiments. VII. Average of twelve experiments. *A* = sperm filtrate from a 5 per cent. suspension. *B* = sperm filtrate from a 2 per cent. suspension. *C* = sea-water control.

Practically no heated sperm reach the surface of the filter mantle as they do not pass through the Whatman filter paper. This might appear to indicate that any active substance is adherent to the heated coagulated sperm and not readily given off into sea-water.



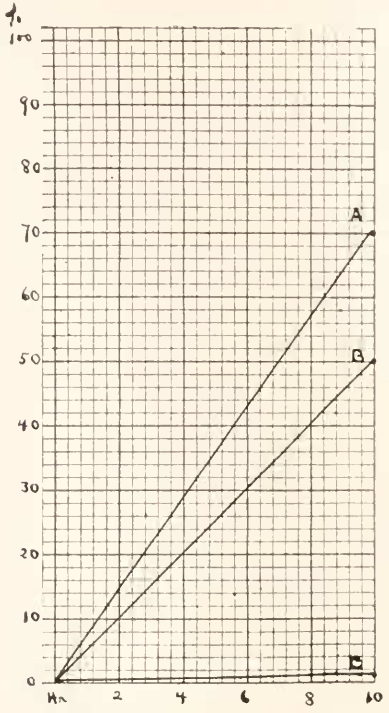
GRAPH VIII. Relation of the strength of the dialyzate to the percentage of ova divided and cytolyzed—*Arbacia punctulata*. *A* = average of three experiments with dialyzates from 50 per cent. sperm suspensions. *B* = average of three experiments with dialyzates from 25 per cent. sperm suspensions. *C* = average of six experiments with sea-water controls. Duration of dialysis = ten hours.

Another filtrate was made by filtering a heated sperm suspension directly through a Mandler filter. As a control part of the same suspension, unheated, was passed through a second filter. The effects of these two preparations are compared in Table III.

The filtrates of the heated sperm suspension have as great an effect on unfertilized ova of the same species as filtrates of fresh suspensions if the suspensions are filtered directly.

The Effect of Boiled Filtrates of Arbacia Sperm.—If the substance which so affects the eggs is an enzyme, heated filtrates may be ineffective. A filtrate prepared from a five per cent. sperm suspension was heated to boiling and then cooled rapidly.

An equal volume of sea-water was treated in the same manner and used as a control. Unheated filtrate and unheated sea-water served as additional controls. The results are given in Table IV.



GRAPH IX. Correlation between the duration of dialysis and the percentage of ova divided and cytolyzed—*Arbacia*. *A* = dialyzate from a 50 per cent. sperm suspension. Duration of dialysis = twenty hours. *B* = dialyzate from a 50 per cent. sperm suspension. Duration of dialysis = ten hours. *C* = sea-water dialyzed for twenty hours against sea-water.

Heating the filtrate as above indicated does not greatly affect its power to produce segmentation and cytolysis of ova of the same species. It should be emphasized that the hydrogen-ion concentration and the osmotic pressure of these solutions are like those of the sea-water controls.

It is thus evident that filtrates and dialyzates of spermatozoa of *Arbacia* contain some substance, not destroyed by heat, which produces profound changes in ova of the same species.

TABLE III.

A COMPARISON OF THE ACTION OF FILTRATES PREPARED FROM HEATED AND FROM UNHEATED SPERM SUSPENSIONS OF *Arbacia* FILTERED DIRECTLY THROUGH MANDLER FILTERS.

Exp.	Filtrate from	Per Cent. of Ova Affected. 2 Hrs.			Per Cent. of Ova Affected. 20 Hrs.		
		Nor.	Abn.	Cyt.	Nor.	Abn.	Cyt.
167	I. Fresh Sperm	98	0	2	0	0	100
	II. Heated Sperm	100	0	0	0	0	100
170	I. Fresh Sperm	62	0	38	0	0	100
	II. Heated Sperm	53	0	47	0	0	100

Nor.—normal undivided ova.

Abn.—undivided ova, abnormal in shape and in the appearance of cytoplasm and nucleus.

Cyt.—cytolyzed. (Partial or complete division precedes cytolysis.)

TABLE IV.

A COMPARISON OF THE ACTION OF HEATED AND UNHEATED SPERM FILTRATES ON OVA OF THE SAME SPECIES—*Arbacia punctulata*.

Time = 14 Hrs.	Per Cent. of Ova Affected. Exp. *A*—III.			Per Cent. of Ova Affected. Exp. *A*—IV.		
Solution.	Nor.	Div.	Cyt.	Nor.	Div.	Cyt.
Sea-water	98	0	2	99	0	1
Heated Sea-water	98	0	2	99	0	1
Sperm Filtrate	62	3	35	74	3	23
Heated Filtrate	84	0	16	81	2	17

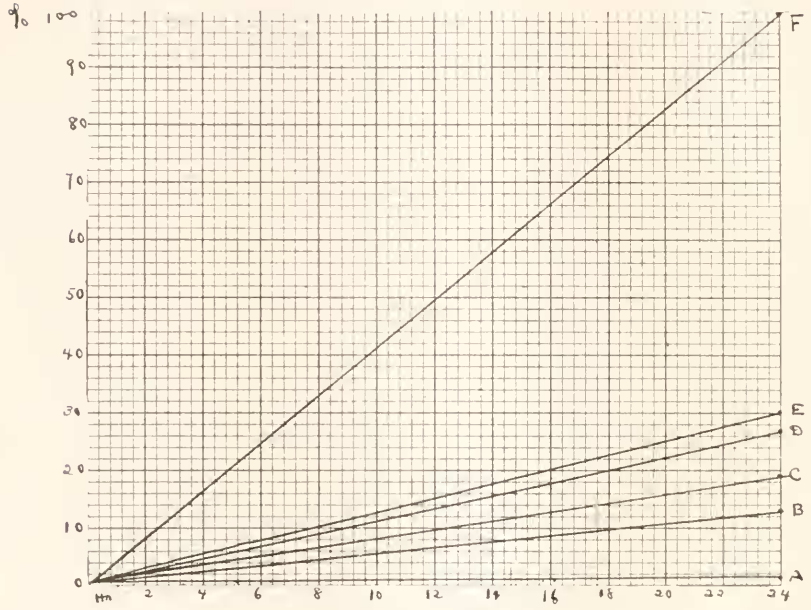
Nor.—undivided and normal.

Div.—divided.

Cyt.—cytolyzed.

b. Strongylocentrotus purpuratus and Strongylocentrotus franciscanus.—The same methods employed with two other species of sea-urchins—*Strongylocentrotus purpuratus* and *Strongylocentrotus*

franciscanus—at Pacific Grove, California, from January to June 1921, yielded similar results. Longer exposures were required than with ova of *Arbacia*. The ova of *S. franciscanus* were the more susceptible to the action of the sperm filtrates, but unfortunately ripe ova of this species were rare during these months



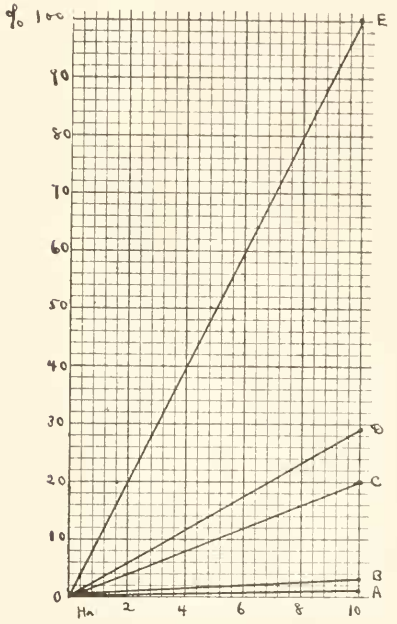
GRAPH X. The effect of limited exposure of ova to sperm filtrates—*Arbacia*. Experiment 156—Observation at the end of twenty-four hours. The percentage of ova divided and cytolyzed is indicated. *A* = sea-water. *B* = exposure to 5 per cent.* filtrate for five minutes. *C* = exposure to 5 per cent. filtrate for ten minutes. *D* = exposure to 5 per cent. filtrate for thirty minutes. *E* = exposure to 5 per cent. filtrate for sixty minutes. *F* = exposure to 5 per cent. filtrate for twenty-four hours.

of 1921 because of an extremely cold season. It is well known that the ova of *S. purpuratus* are resistant to parthenogenetic agents, Loeb (1916, pp. 99–103). The hope that the conditions of low temperature (12°–15° C.) and high hydrogen-ion concentration (p_H 7.6–7.8) prevailing at Pacific Grove might favor normal segmentation and normal development of ova treated with sperm filtrates was not realized. Yet, as will be demonstrated in later experiments, a brief exposure to such filtrates

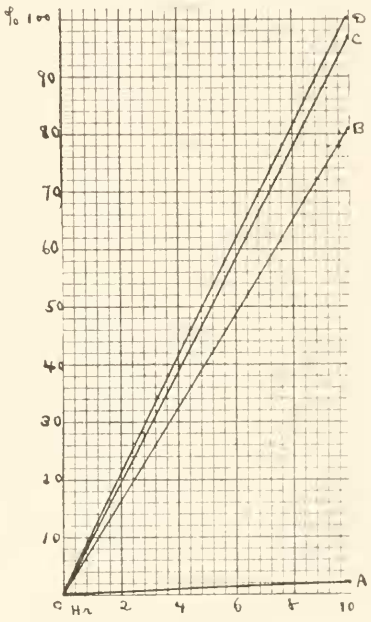
* 5 per cent. filtrate = a filtrate from a 5 per cent. suspension.

produced decided changes in ova of both species of *Strongylocentrotus*.

c. Nereis limbata.—Sperm filtrates of *Nereis limbata* produce effects on ova of this species comparable to those produced by foreign sperm filtrates. An exposure of *Nereis* eggs to filtrates



GRAPH XI.



GRAPH XII.

The effect on ova of *Arbacia* of limited exposure to filtrates.

Filtrate of a 2 Per Cent. Suspension.	Filtrate of a 2 Per Cent. Suspension.
A = sea-water 10 hrs.	*A* = sea-water 10 hrs.
= filtrate 15″–45″	= filtrate 15″–45″
B = " 75″	*B* = " 210″
C = " 135″	*C* = " 4.5 hrs.
D = " 195″	*D* = " 9 hrs.
E = " 9 hrs.	

The percentages of ova divided and cytolyzed are indicated.

made from one, two or ten per cent. *Nereis* sperm suspensions results in the formation of jelly and fertilization membranes and the complete maturation of a certain percentage of eggs. The most satisfactory results were obtained with eggs transferred directly to a two per cent. filtrate, and within two hours after

the *Nereis* were collected. In this experiment the majority of the eggs matured. The maturation was almost as rapid as in fertilized eggs but the subsequent segmentation and development was slow and abnormal. Segmentation into two, four, and eight cells was observed and verified for me by Dr. Alvalyn Woodward. Free swimming trochophores developed which were abnormal in shape, internal structure, and in the distribution of cilia. In no case did such changes occur in the sea-water controls. Similar results were obtained with four sperm filtrates of *Nereis limbata*.

Summary of the Effects of Sperm Filtrates and Dialyzates on Ova of the Same Species.—Sperm filtrates and dialyzates of *Arbacia punctulata*, and sperm filtrates of *Strongylocentrotus purpuratus*, of *Strongylocentrotus franciscanus*, and of *Nereis limbata* produce marked changes in ova of the same species. These are characterized by changes in form; nuclear and cell division; and, with the exception of *Nereis limbata*, by subsequent fragmentation and cytolysis. "Fragmentation" is a separation of the segments due possibly to a lack of a fertilization membrane. In *Nereis limbata* the sperm filtrates cause maturation; formation of fertilization membranes; segmentation; and, in a low percentage of cases, the development of abnormal larvæ.

V. Filtrates as Factors in Parthenogenesis.

It seemed possible in view of the results obtained by Loeb (1905) and others that the ova of sea-urchins, treated with filtrates of sperm, might develop perfectly if they were subsequently exposed for a brief period to Loeb's "hypertonic sea-water". In Loeb's experiments, exposure of unfertilized ova of sea-urchins to hypertonic sea-water, at the temperature prevailing during the breeding season, produced development in a very small percentage of the ova. Fertilization membranes did not form usually and the developing plutei did not swim at the surface of the water as did normal plutei. A brief preliminary treatment of the ova with a very dilute solution of butyric acid increased the percentage of ova which developed, and the latter were normal in macroscopic structure and in their reactions. Recently Just (1922), employing a greater concentration than

that used by Loeb, has succeeded in producing healthy viable plutei of *Arbacia punctulata* by exposing ova of this species to hypertonic sea-water alone. In view of these results it may be that the butyric acid sensitizes the eggs to the action of the hypertonic sea-water. Experiments were undertaken to determine whether or not sperm filtrates may also act as sensitizers.

The procedure employed in this phase of the investigation was as follows: unfertilized eggs of *Arbacia* were exposed for periods of one minute to two hours to a sperm filtrate and subsequently for twenty to thirty-five minutes to Loeb's hypertonic sea-water. A control series consisted of ova exposed first to sea-water and then to Loeb's hypertonic sea-water. One set of unfertilized and one set of fertilized eggs served as controls for each experiment.

In the majority of experiments no development took place in the ova exposed to hypertonic sea-water only. When such an exception occurred, the percentage of ova which developed was small and the plutei did not swim at the surface of the water. In every experiment many of the ova subjected to the double treatment, *i.e.*, to the filtrate followed by the hypertonic sea-water, developed as far as the blastula and gastrula stages. A small percentage developed into perfect plutei which swam at the surface. The majority cytolyzed. Because of the high percentage which cytolyze at the time when the blastulæ and gastrulæ form, it is impossible to determine the exact percentage which develop to these stages. The sea-water containing them was centrifuged and the residue examined. An effort was made to ensure the transfer of equal quantities of eggs and of sea-water in each experiment, and equal periods of centrifuging were employed to ensure equal possibilities of precipitation of ova or of developing individuals. The results of these experiments are given in Table V.

As already indicated the sperm filtrates rarely cause membrane formation. In the few instances where these form, they are thin and but slightly elevated. In one filtrate such membranes developed on ova after an exposure to the filtrate for thirty to thirty-five minutes. Of the ova remaining in the filtrate for one to fifteen minutes and subsequently transferred to hypertonic

sea-water for twenty to thirty-five minutes, a larger percentage developed than in other experiments. In this series no ova developed after exposure to hypertonic sea-water alone. In Table VI. the results of these experiments are summarized. The figures indicate approximate percentages.

Table V.

A Comparison of the Number of Ova of *Arbacia* which Develop after Treatment with Filtrate Followed by Exposure to Hypertonic Sea-water.

Time of observation and total count = 70 hrs.

Transfer from	Sea-water.			Hypertonic S.W. 30″			Hypertonic S.W. 35″		
	B.	G.	P.	B.	G.	P.	B.	G.	P.
Sea-water 15″	0	0	0	1	0	1	2	0	0
Filtrate									
2″	0	0	0	13	0	5	56	0	28
10″	0	0	0	0	0	2	41	0	14
15″	0	0	0	74	0	0	36	0	3

The letters B, G, and P indicate blastulæ, gastrulæ, and plutei respectively. The figures given here indicate the number, not the percentage, of developing ova.

A comparison of the effects of the preliminary treatment with sea-water and with sperm filtrates reveals, in the latter experiments, a slight increase in the percentage of ova which develop into plutei perfect in macroscopic appearance and in reactions; and a decided increase in the percentage which cytolyze after exposure to hypertonic sea-water. The preliminary treatment with the sperm filtrate apparently sensitizes the ova to the action of the hypertonic sea-water.

Tests were also made to determine whether the sperm filtrates increase the susceptibility of the ova of *Strongylocentrotus purpuratus* to the action of hypertonic sea-water. Certain preliminary experiments were performed to familiarize the writer with the effect of hypertonic sea-water alone on the ova of this species. Ova were exposed to it for periods of thirty minutes to four hours. The effect was tested by transferring them to sea-

Table VI.

The Effect Produced on Ova of *Arbacia punctulata* by Sperm Filtrates of the Same Species Followed by Hypertonic Sea-water.

Transfer from	To Hypertonic S.W. 20″.				Hypertonic S.W. 25″.				Hypertonic S.W. 30″.				Hypertonic S.W. 35″.			
	Nor.	Div.	Dev.	Cyt.	Nor.	Div.	Dev.	Cyt.	Nor.	Div.	Dev.	Cyt.	Nor.	Div.	Dev.	Cyt.
	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%	%
Sea-water 15″	95.8	0	0	4.2	91	1	0	8	90	0	0	10	90	0	0.5	9.5
Filtrate 1″	75.5	0	0	24.5	53.6	0	5.7	41.7	75	0	4.5	20.5	27.5	0	1.5	71
5″	0	0	0	100	75	0	3.8	21.2	62	0	3	35	28.3	0	2	69.7
10″	66	0	3.4	30.6	8.8	0	0	91.2	21.8	0	6.1	72.1	23.6	0	0.4	76
15″	48	0	2.7	49.3	14	0	1	85.0	3	0	0	97	1	0	0	99

water and to inseminated sea-water. Ova treated for thirty minutes to two hours were uninjured. Longer exposures caused cytolysis of ova transferred to sea-water and prevented normal fertilization and development of ova subsequently inseminated in sea-water. The percentage of the ova affected bore a direct relation to the duration of the exposure to the hypertonic sea-water.

A repetition of Loeb's method of producing parthenogenesis in this species demonstrated that treatment with 1.5 per cent. butyric acid for one minute followed by hypertonic sea-water for one hour led to development of the majority of the ova. Other experiments indicated that a slight decrease in hydrogen-ion concentration (*e.g.*, p_H 8.3–8.7) facilitated the process. In place of the butyric acid ova were given a preliminary treatment with sperm filtrates in the hope of obtaining development, or of intensifying the action of the hypertonic sea-water. The results at the end of eighteen hours are indicated in Table VII.

Table VII.

Effect of Exposure to Filtrate Followed by Hypertonic Sea-water on Ova of *Strongylocentrotus purpuratus*.

Exp.	Prep.	% Filt.	Duration of Exposure.		Effects on the Ova.			
			To		Percentage.			
			Filtrate	Hypertonic S.W.	Nor.	Abn.	Div.	Cyt.
83	1/26	10	0″	70″	99	0	0	1
			30″	70″	98	0	0	2
			60″	70″	98	0	0	2
			120″	70″	66	0	1	33
			30″	120″	99	0	0	1
			60″	120″	0	97.5	0	2.5
			120″	120″	0	0	0	100

It has already been mentioned that ova of *Strongylocentrotus purpuratus* are more resistant to parthenogenetic agents than are those of *Arbacia punctulata*. In these experiments ova of the former species, exposed either to hypertonic sea-water alone

or to filtrate and hypertonic sea-water, failed to develop. Occasionally atypical membranes formed after the double treatment and irregular division ensued. It is of interest that ova which had been exposed to the filtrate for two hours did not shrink when transferred to hypertonic sea-water, as did those transferred directly from sea-water to the hypertonic sea-water. This would seem to indicate that the permeability of the ova exposed to the filtrate had increased. Another indication of the effect of the filtrate in these experiments follows: Ova given the double treatment of filtrate followed by hypertonic sea-water were less capable of normal fertilization and development than those exposed to hypertonic sea-water alone.

Summary of the Function of Filtrates as Factors in Parthenogenesis.—Exposure of ova of *Arbacia punctulata* or of *Strongylocentrotus purpuratus* to sperm filtrates of the same species produce changes which render them more susceptible to the action of hypertonic sea-water. This is indicated in *Arbacia* by a larger percentage of development and of cytolysis; and in *S. purpuratus* by a greater tendency of ova to cytolyze or to lose their power of normal fertilization and development if exposed to both filtrate and hypertonic sea-water.

VI. Filtrates as Factors in Fertilization.

The increase produced by sperm filtrates in the susceptibility of *Arbacia* ova to parthenogenetic agents, and the apparent increase in permeability of ova of *S. purpuratus* suggest that such preparations may also facilitate fertilization.

An attempt was made to obtain "resistant" eggs early in the breeding season in order to try the effect of fertilizing such ova in sperm filtrates. During two seasons such "resistant" eggs were not found. The ova obtained were either immature, as indicated by their failure to develop if fertilized with a small or large amount of sperm suspension, or else ripe. In the latter case no increase over the usual percentage of development could be expected.

Conditions leading to increase in permeability of mature ova tend to allow the entrance of more than a single spermatozoön when such eggs are inseminated. This polyspermy usually

results in abnormal development. It may occur if mature ova are inseminated in sperm filtrates. To determine this, two milliliters of washed *Arbacia* eggs were placed in twenty-five milliliters of sperm filtrate and, as a control, a similar quantity in twenty-five milliliters of sea-water. These were inseminated immediately, or after exposure to the filtrate or sea-water for from one minute to four hours. Frequent observations were made to determine whether the filtrates affect either fertilization or development. The effects observed are as follows in the ova fertilized in the filtrate and allowed to develop in it: lack of a normal fertilization membrane; abnormal changes in shape; a low percentage of dividing ova; abnormal division; delayed and abnormal development; and cytolysis. In the controls normal fertilization and development occurred. Similar results were obtained in thirteen series of experiments in which several filtrates were used. Gemmil (1900) describes similar conditions resulting from heavy insemination and finds them accompanying polyspermy as demonstrated by histological examination.

The abnormal development which occurs when ova are inseminated in the sperm filtrates may be a result of an abnormal fertilization reaction. If this is due to changes in the ova in the filtrate, it may be that such changes are reversible if the exposure to the filtrate is of short duration. Ova of *Arbacia punctulata* were exposed to filtrates made from two per cent. suspensions for from one minute to two and a half hours, and to filtrates from five per cent. suspensions for one to thirty minutes. Some were inseminated in the filtrates and others after transfer to sea-water. Few of those inseminated in the filtrate developed normally. The majority of those inseminated after transfer to sea-water formed fertilization membranes and developed normally. The changes produced in the ova by the filtrates, which tend to prevent normal reactions between ova and sperm, are reversible if the period of exposure is brief. Prolonged exposure to filtrates, however, wrought such changes in the majority of ova that they lost their capacity for fertilization and development completely.

Ova of *Strongylocentrotus purpuratus* exposed for short periods to sperm filtrates may not exhibit any change in optical appear-

ance or in shape yet may be so influenced that after transfer to sea-water they fail to develop normally if inseminated with fresh sperm. Such was found to be the case in eighteen experiments in which ova were exposed to the action of a number of filtrates and subsequently inseminated in sea-water. Although the sperm were active in the filtrates, few membranes formed and these were abnormal in that they were irregular and but slightly raised from the surface of the egg. Subsequent divisions were irregular; development was slow and abnormal; and the majority of ova cytolyzed later. Gastrulæ formed in a few, but these lacked an enteron, and plutei were irregular in shape with thickened areas not normally present. These resembled plutei obtained when ova are inseminated with a large excess of sperm when polyspermy is known to occur.

A correlation between the reversibility of changes in ova produced by abnormal constituents in sea-water and the duration of their exposure to these substances has been recorded by Loeb (1915) for butyric acid and hypertonic sea-water; by Lillie (1921) for copper; and by Clowes and Smith (1923 and 1924) for hydrogen ions. In some instances the change is of such a nature that it acts as a block to the entrance of sperm; in others it permits polyspermy. Just (1923) found that eggs of *Echinarachnius* fertilized in blood, though they fail to develop, nevertheless take in sperm.

As stated by Clowes (1924) "it is difficult to distinguish polyspermic from abnormally dividing eggs without cytological examination." Such a study of ova of *Arbacia* inseminated in the sperm filtrates reveals that polyspermy occurred in many of the ova.

The interference with the fertilization process may be due in part to injury to the sperm caused by the sperm filtrate. However, examinations of suspensions of sperm in filtrates reveal that the sperm remain active in such suspensions for hours. Furthermore if such sperm are used for insemination of ova in sea-water, they will effect normal fertilization and development. The results tabulated below indicate that the sperm are not injured by an exposure of one hour either to sea-water or to a sperm filtrate in the concentrations employed; but that ova

were affected by a similar duration of exposure to some of the same filtrate.

TABLE VIII.

A COMPARISON OF THE ACTION OF FILTRATES ON THE FERTILIZING CAPACITY OF SPERM AND OF OVA OF *Strongylocentrotus purpuratus*.

Exp.	Prep.	Filt.	Expos.	Ova from Sea-water.				Ova from Sperm Filtrate.			
73	12/28/11	10%		% Nor.	% Div.	% Abn. Div.	% Cyt.	% Nor.	% Div.	% Abn. Div.	% Cyt.
Sperm exposed to sperm filtrate..			0″	0	90	10	0	0	90	10	0
			1″	0	94	3	3	0	59	35	6
			5″	0	95	5	0	0	85	10	5
			10″	0	91	5	4	0	68	29	3
			20″	0	95	3	2	0	56	43	1
			30″	0	96	3	1	3	23	18	56
			60″	0	98	2	0	0	0	30	70
Sperm suspension in sea-water....			30″	0	98	1	1	0	0	33.4	66.6
			60″	0	96	0	4	0	0	12	88

It is also possible that filtrates may interfere with the development of fertilized ova. This proved to be true. The ova of *Arbacia punctulata* and of *Strongylocentrotus purpuratus* transferred ten minutes after insemination in sea-water to sperm filtrates failed to develop normally, and within eighteen hours the majority had cytolyzed.

Summary of the Action of Filtrates as Factors in Fertilization.— Sperm filtrates produce changes in the eggs of the same species which interfere with a normal fertilization reaction if the eggs are inseminated in the filtrate. The changes produced by a brief exposure are reversible; by a longer exposure irreversible. Such filtrates prevent normal development of eggs previously inseminated in sea-water. The changes are of such a nature that the entrance of sperm is facilitated and polyspermy results.

VII. TEST OF FILTRATES FOR A "SPERM-FERTILIZING AND AGGLUTINABLE SUBSTANCE."

Lillie (1919) has suggested that the spermatozoa bear a fertilizing substance identical with the agglutinable substance

which is apparently lost by spermatozoa in staling in sea-water. If this is present in sea-water filtrates and dialyzates, it should combine with the "agglutinin" of "egg-water." This was tested as follows. The agglutinating unit strength of *Arbacia* egg-water was first determined, Lillie (1914). Dilutions of such egg-water with sperm filtrates and with sea-water were compared as to their power to agglutinate fresh sperm suspensions and no difference could be detected in the capacity of the two sets of dilutions to agglutinate sperm. Either an insufficient amount of the combining substance is present in the sperm filtrates or the substance or substances present do not have the power to combine with the agglutinin of the egg-water.[4]

The agglutinin does not pass through collodion sacs. If the substance in sperm dialyzates is the substance postulated by Lillie, egg-water dialyzed against a sperm suspension should lose its agglutinating power more rapidly than a similar egg-water dialyzed against sea-water. In experiments devised to test this theory no difference in the rate of loss of agglutinin could be detected.

The "fertilizin" of Lillie in *Arbacia* egg-water may, according to Woodward (1918), consist of two parts: an agglutinating and an activating substance. The latter only passes through a Mandler filter. This activates sperm of the same species, and has the power of causing parthenogenetic development of ova of *Nereis limbata*, Sampson (unpublished, quoted by Woodward) and Woodward (1921). If its action is intensified by a fertilizing substance given off by sperm, a combination of filtrates of sperm and of egg-water should be more effective than either alone. No such intensifying effect could be demonstrated in any of the experiments devised to test this possibility.

VIII. Discussion.

In *Nereis limbata* and in sea-urchins the fertilizable period of the gametes is short. Causes for the brevity of this period have been discovered for ova but not for spermatozoa, as indicated by Lillie and Just (1924) in their recent survey of the subject.

[4] It should be recalled that some of the sperm remaining on the surface of the filter mantles and in the dialyzing sacs are agglutinable and retain their fertilizing power at the end of the periods of filtration and of dialysis.

The fact that loss of fertilizing power of the sperm occurs rapidly and precedes loss of motility has suggested the idea that the spermatozoön carries a fertilizing substance which may be lost in sea-water; and this has led to various attempts, of which this is the most recent, to isolate such a substance and to produce development of ova of the same species with it. In this investigation filtrates and dialyzates of sperm suspensions in sea-water have been obtained which initiate development of specific ova, although the development is incomplete.

It is not surprising to find that concentrated sperm suspensions exposed to egg-secretions of a foreign species, that extracts of cells, egg-secretions, and blood cause cytolysis of alien ova, "since it is recognized that something present in mammalian blood serum cytolyzes cells of unrelated animals," Loeb (1913). Sperm killed by heat, extracts of cells, and blood serum have no activating effect on ova of the same species. Specific egg-secretion ("egg-water") has no effect on ova of *Nereis*, but according to Glaser (1915) and Woodward (1918) does produce incomplete activation of ova of *Asterias* and *Arbacia*. Prolonged exposure of ova is necessary, and Lillie and Just (1924) have suggested that there are extraneous parthenogenetic factors present in the egg-water employed.

Careful tests of all the sperm filtrates and dialyzates used in this investigation indicate that the preparations do not contain living sperm or fragments of them. The ova employed are not normally parthenogenetic. Controls give evidence that no substance derived from filters or dialyzers is accountable for the results obtained; and factors which might produce parthenogenesis: abnormal specific gravity, abnormal hydrogen ion concentration, and excess of carbon dioxide, are lacking. The preparations contain carbon but insufficient nitrogen to be detected even by microchemical methods. No lipolytic enzyme could be detected. The preparations are not colloidal; and all attempts to obtain precipitates from them by means of alcohol or the reagents used by Robertson (*loc. cit.*) and Woodward (*loc. cit.*) failed. Sperm filtrates and dialyzates activate ova rapidly and this property is not destroyed by boiling. The effect produced is evidently due to a special physiological activator derived from sperm of the same species.

Experiments to determine whether the substance or substances present in filtrates and dialyzates are "tissue specific," acting only on ova, have not been undertaken. It is true that they do not cytolyze species sperm, as the latter retain their fertilizing power after prolonged exposure to such preparations. The latter are not "species specific" since they readily activate ova of unrelated species. Tests have not been performed to determine whether the effect on species and on foreign ova is due to the same constituent of the preparations.

If the substance acts as a superficial cytolytic agent as suggested by Loeb (1916), it is to be expected that membrane elevation or swelling will occur in ova of the same species exposed to the sperm filtrates and dialyzates. Such occurred in *Nereis* only. However, such preparations cause partial activation and changes in the protoplasm of sea-urchin ova; and there is evidence that the properties of the egg surface are affected. Thus, after exposure to sperm filtrates, unfertilized ova of sea-urchins are more susceptible to the action of hypertonic sea-water; are in a condition which facilitates polyspermy; and their permeability is increased. Fertilized eggs, transferred to such preparations within ten minutes after insemination in sea-water, fail to develop normally. This may be due in part at least to an increase in the permeability of the egg surface. The sperm are uninjured by long exposure and the changes in the ova are reversible if the duration of exposure to such preparations is brief.

According to the "fertilizin" theory of Lillie (1914), substances ("receptors") given off by sperm activate "fertilizin" an essential constituent of the cortex of mature eggs. This in turn initiates the development of the egg. If "receptors" exist in active form and in sufficient quantity in these sperm filtrates and dialyzates, the latter should produce the following effects: initiation of development of mature ova of the same species; such activation of "fertilizin" in egg-water as to make the latter an efficient parthenogenetic agent; such combination with an agglutinating substance in egg-water as to destroy the power of the latter to agglutinate fresh sperm suspensions. The first of these results only has been obtained in this investigation. If

sperm receptors are released more readily in specific egg-water, filtrates of sperm suspensions in egg-water may evince greater activating power than a combination consisting of a sperm filtrate and filtered egg-water. Tests gave no indication of any difference between them. It is also possible, as suggested by Lillie (1915), that other substances are extracted from sperm which may tend to neutralize the activating substances released by them.

There is abundant evidence that the sperm in contact with mature ova, or secretions of ova, of the same species undergo changes which are essential for fertilization. Their metabolism is increased as indicated by their increased motility; their chemical composition is changed as indicated by decrease in refringibility, by swelling of the sperm head, by changes in viscosity, and by surface changes which permit agglutination to occur. Spermatozoa may enter unripe eggs which lack "fertilizin" or mature eggs from which it has been removed experimentally but the sperm are not changed and they do not activate the eggs.

It is significant that in order for fertilization to occur *these essential changes in the spermatozoön must be produced when the latter is in close proximity to an ovum.* Sperm which have received a long exposure to sea-water or a brief exposure to egg-water are active and may surround or even enter ova of the same species, yet fail to fertilize them. This loss of fertilizing capacity, the transitory nature of agglutination, and the inability to obtain a second agglutination reaction with sperm are indications of a loss of substances essential in fertilization. To effect perfect development *such substances must be concentrated at the surface of the sperm head at the instant when the latter comes into contact with an ovum.* Under such conditions they may initiate a chain of chemical reactions, starting in the cortex of the ovum and eventually involving all parts of the protoplasm of the egg, *i.e.*, they may activate "fertilizin." If released into sea-water or egg-water normally, or under the experimental conditions of filtration and dialysis, these substances may be unable to produce complete activation of ova of the same species, because of dilution, instability, or neutralization by other substances elimi-

nated or extracted from sperm. They may, however, produce changes in the surface of the ovum which facilitate its reaction with a spermatozoön. These changes may account, in part, for polyspermy when ova are inseminated with an excess of fresh sperm and for the effectiveness of stale sperm if concentrated suspensions are used. Such substances may account for the antagonistic effect produced by sperm of one species on those of another; and for the neutralizing effect of concentrated sperm suspensions on the action of blood serum.

IX. Summary.

1. Solutions obtained by filtration and dialysis of suspensions of living sperm in sea-water activate ova of the same species.

2. Tests indicate that the effect is produced by some substance derived from the sperm, and not by some extraneous parthenogenetic factor.

3. Ova of *Nereis* exposed to specific sperm filtrates form fertilization membranes, complete their maturation and some develop into abnormal trochophores. Ova of sea-urchins fail to form membranes but do undergo nuclear and cell division.

4. Ova exposed to filtrates and dialyzates are rendered more susceptible to the action of hypertonic sea-water, and to the entrance of sperm.

5. In normal fertilization sperm exposed to "fertilizin" undergo profound modification in chemical structure and organization; and unless such modification occurs the sperm fail to fertilize the egg, even though they may enter it. It is possible that substances localized in the surface of the sperm head activate the ovum. Such localization is transitory.

6. Such substances, when given off by sperm into sea-water or egg-water, either because of dilution, decomposition, or admixture with waste products given off by sperm, produce definite changes in such ova, but are unable to effect complete activation of ova of the same species.

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